Patent Application
Docket No. GJE-30
Serial No. 09/297,486

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Richard A. Schnizer
Art Unit	:	1632
Applicants	:	John Francis Martin, Seppo Yla-Herttuala, Stephen George Edward Barker
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For	:	Therapeutic Use of an Agent that Stimulates NO or Prostacyclin Production and Delivery Device

4/1/03

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF JOHN FRANCIS MARTIN, M.D. UNDER 37 CFR §1.132

Sir:

I, John Francis Martin, M.D., hereby declare:

THAT, I am a co-inventor of the subject matter claimed in U.S. patent application Serial No. 09/297,486 (hereinafter the '486 application);

THAT, I have read and understood the '486 application;

THAT, I have read and understood the rejection of claims in the Office Action mailed November 16, 2001 in the '486 application;

AND, being thus duly qualified, do further declare:

The claims of the subject application are rejected under 35 USC §112, first paragraph, as nonenabled. The Examiner indicates that the subject specification does not enable the claimed methods for treating any vascular disorder in species other than rabbits. I respectfully assert that the specification does enable the pending claims. Attached as Appendix A of this Declaration are results from our studies presented to the Recombinant Gene Advisory Committee of the National Institutes of Health and the Food & Drug Administration that show the successful delivery and expression of a DNA expression vector encoding a VEGF receptor agonist in target cells of blood vessels for the treatment and

prevention of conditions, such as intimal hyperplasia or those associated with NO stimulation or prostacyclin production, in species other than rabbits.

In the studies that we carried out, the carotid artery in fifty-two pigs was anastomosed to the ipsilateral internal jugular vein. As seen in Table 7.1 of the study in attached Appendix A, the site of anastomosis in thirteen groups of pigs (four pigs per group) was treated with an adventitial device of the '486 application containing different doses of a DNA expression vector containing a VEGF-D ("Trinam" is VEGF-D) transgene to produce cell transfection at the anastomosed site. Certain groups of pigs were treated with saline injected wrap devices as a control and certain groups were treated with a LacZ marker gene to assess the biodistribution of the DNA expression vector. Appropriate samples were collected to perform PCR, RT-PCR, and immunohistochemical analyses to determine the biodistribution and expression of the VEGF-D transgene in different tissues and assess immune responses. Ultrasound, clinical monitoring, biochemical and haematological analysis, microscopic analysis, and immunocytochemical analysis were conducted to assess the efficacy of transgene expression.

The PCR results showed that seven out of eight samples were PCR positive when administered the highest doses (10^9 and 10^{11} viral particles) of the DNA expression vector. RT-PCR analysis of VEGF-D transgene expression at the site of anastomosis where high concentrations of the DNA vector encoding VEGF-D were administered also displayed transgene expression.

Ultrasound analyses were performed at day 7 and day 28 to measure the degree of graft patency (stenosis) at the anastomsed site. Results from the ultrasound analyses can be seen in Tables 7.4 and 7.5 of the study in attached Appendix A and suggest that half of the animals that received the highest dose of the DNA vector encoding VEGF-D exhibited less than fifty-percent (50%) stenosis. As indicated in the study results, it is generally accepted that stenosis of less than 50% does not cause hemodynamic changes and, therefore, is regarded as insignificant in relation to flow restriction. In addition, microscopic evaluation of the site of anastomosis was performed to assess thrombus, smooth muscle cell, and endothelial proliferation, intimal proliferation, mural haemorrhage and thickening, medial smooth muscle cell proliferation, adventitial granulation tissue/fibroblast proliferation, perivenous haemorrhage/fibrinoid material, inflammatory cell infiltration, and granulation tissue/fibroblast proliferation. Results from these microscopic evaluations suggest the

treatment with high doses of a DNA vector containing a VEGF-D transgene provide a biological effect by improving endothelial function inhibiting thrombosis.

It is well understood in the art that changes that occur in the cardiovascular system of the pig are indicative of changes that occur in the human cardiovascular system, both in terms of physiology and pathophysiology. The Food & Drug Administration has indicated that results from pig studies as suitable to the criteria in the process for obtaining approval to perform clinical studies on humans. Thus, from our evidence showing the successful delivery and expression of a DNA expression vector encoding a VEGF agonist in pigs, the skilled artisan would conclude that the subject DNA expression vector encoding VEGF agonists could be used in the treatment of conditions, such as intimal hyperplasia, across species, including humans.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further declarant sayeth naught.

Signed: _____

John Francis Martin, ~~Ph.D.~~ M.D, F. Med. Sci.

Date: 16.9.2.

APPENDIX A

Study 6: Trinam: Evaluation in a pig model of the human vascular access situation

Following discussions with CBER, a pre-clinical study in pigs was performed to assess the safety and efficacy of delivering a transgene for human vascular endothelial growth factor D (VEGF-D,) using a fist generation adenoviral vector, and a perivascular collagen collar, which was designed to localize transduction to the target cells.

□ ***Methodology:***

The study involved using a pig model which was designed to mimic the surgery used to provide vascular access for haemodialysis in humans. In summary, this model involved using a PTFE loop-graft to join the porcine carotid artery to the ipsilateral internal jugular vein. A perivascular collar was then applied at the site of the graft vein anastomosis and different concentrations of adenovirus containing either the VEGF-D transgene or the LacZ marker gene were then injected into the collar space. As a control, saline was also injected into the collar of three groups of animals. Following injections, the different groups of pigs were euthanased at different time points as shown in Table 7.1 below.

During the 'in-life' phase of the study, the blood flow through the graft was measured by ultrasound 24 hours after treatment and also on days 7 and 28. In addition, the general welfare and body weight of the animals was assessed. At the time of autopsy, the appropriate samples were collected to allow the following parameters to be assessed; degradation of the collagen collar, a comprehensive evaluation of biochemical and haematological parameters, and macroscopic, microscopic and histological analysis of the tissue samples. In addition, samples were collected for PCR, RT-PCR and immunohistochemical analysis to determine the bio-distribution of the adenoviral vectors and expression of the transgene in different tissues and immune responses against the adenovirus, or collagen collar.

Table 7.1: The treatment regimes for the different groups of pigs.

Group No.	1 n=4	2 n=4	3 n=4	4 n=2	5 n=2	6 n=4	7 n=4	8 n=2	9 n=2	10 n=4	11 n=4	12 n=2	13 n=2
VEGF-D viral particles	10^7	10^9	10^{11}	–	–	10^9	10^{11}	–	–	10^9	10^{11}	–	–
LacZ viral particles	–	–	–	10^9	–	–	–	10^9	–	–	–	10^9	–
Saline	–	–	–	–	+	–	–	–	+	–	–	–	+
Time of sacrifice (days post treatment)	3	3	3	3	3	14	14	14	14	60	60	60	60

□ ***Results***

Transfection efficiency and expression of the transgenes: The efficiency of transfection of the adenoviral vectors at the site of administration was assessed by PCR. The VEGF-D PCR results for the samples collected from the site of administration in the animals sacrificed after 3 days, showed that at the two highest doses (10^9 and 10^{11} viral particles), seven out of eight of the samples for each group (two per animal, four animals per group) were PCT positive. In contrast, in the group that received the lowest dose of virus (10^7 viral particles), only 2 out of 8 of the samples were positive. At day 14, only 50% of the samples analysed from the site of the anastomosis were positive in the animals that received 10^9 viral particles, whilst 6 out of eight samples were positive in the animals that received 10^{11} viral particles. At day 60, 6/8 and 7/8 samples from the site of anastomosis in the animals treated with 10^9 and 10^{11} viral particles were positive respectively.

The bio-distribution of the vector was also assessed by PCR. Analysis of the tissue samples collected from the major organs and reproductive tissues showed that there was a wide distribution of the vector outside the area of treatment. At the day three time point, the number of positive tissues appeared to increase with dose. For example; twelve tissues outside the area of treatment were positive at the 10^7 and 10^9 doses. In contrast at the top dose (10^{11} viral particles), 26 of the 47 tissues tested were PCR positive. Positive samples were detected in all the major tissues examined; spleen, liver, lung, kidney, skeletal muscle, as well as the eyes and gonads. At day 14, there was no significant difference between the number of positive tissues in the samples collected from the animal treated with the two highest doses. At day 60, only 9/48 of the tissue samples from outside the site of

anastomosis were positive in samples from the pigs that received the top dose, whilst 25/51 of the samples were positive at the dose of 10^9 viral particles

Analysis of the urine, bile and blood samples that were collected at the time of sacrifice, from all the animals, showed that no adenovirus could be detected in any of the samples that were tested.

RT-PCR analysis of VEGF-D expression using samples collected at the site of the anastomosis from the animals sacrificed at day 3, showed that the transgene was expressed in pigs that had been treated with the two highest doses of adenovirus. For example, in the animals that received 10^9 viral particles, 50% of the samples were positive, whilst 75% of the samples were positive in the animals that received 10^{11} viral particles. In contrast, no positive samples were detected in the animals that received the lowest dose of virus (10^7 viral particles). Only four RT-PCR positive samples were detected in the 32 samples collected from the site of treatment in the animals that were sacrificed after 14 and 60 days. These results indicate that, as expected, expression of the transgene was transient.

Expression of the transgene in tissues outside the target site of was also assessed. The results showed that expression was limited predominately to the target site. In total, only 7 (4 samples from the carotid artery, 1 sample from the forebrain, one spleen and 1 kidney sample) out of 514 tissues analyzed were RT-PCR positive in the tissues that were collected at either day 3 or 14. No long-term expression of the transgene was detected in any of the samples collected from outside the area of administration of the virus in the animals killed at day 60. Similarly, expression of the transgene was not detected in any of the samples analyzed from the eye or the gonads at any of the time points.

X-gal staining of the samples from the animals treated with the LacZ marker gene was used to assess the transfection efficiency. The results showed that the transfection efficiency was less than 1%. Positive results were observed in samples collected from the site of anastomosis at day 3 and 14, but no positive samples were detected in equivalent samples from the animals killed at day 60. In contrast to the VEGF-D RT-PCR results, no expression was detected in any tissues outside the site of the anastomosis.

Efficacy of VEGF-D expression: The efficacy of Trinam was assessed by two different methods: ultrasound and microscopic evaluation of the site of the anastomosis.

Ultrasound analysis was used to measure the degree of graft patency (stenosis), at the site of anastomosis, at either 24 hours, seven days or 28 days post surgery. If the graft was not found to be patent at 24 hours they were replaced by another animal. This was because loss of patency at such a short time interval after surgery was considered to be a surgical failure and not related to any pathological sequelae. The ultrasound results for the day 7 and day 28 measurements are shown in Tables 7.4 and 7.5 respectively.

Table 7.4: Measurement of the percentage of stenosis in the different groups of pigs at day 7.

Dose	A No stenosis	B ≤50% stenosis	C >50-≤75% stenosis	D Blocked or > 75% stenosis
Trinam 10^{11} (n = 8)	3	1	2	2
Trinam 10^{9} (n = 8)	1	2	2	3
LacZ (n = 4)	0	1	1	2
Saline (n = 4)	0	1	1	2

Table 7.5: Measurement of the percentage of stenosis in the different groups of pigs at day 28.

Dose	A No stenosis	B ≤50% stenosis	C >50-≤75% stenosis	D Blocked or > 75% stenosis
Trinam 10^{11} (n = 8)	1	1	1	1
Trinam 10^{9} (n = 8)	0	0	0	4
LacZ (n = 4)	0	0	0	2
Saline (n = 4)	0	0	0	2

The day 7 ultrasound results, as shown in Table 7.4, show that only 5/16 of the pigs which received the LacZ transgene, the saline solution or 10^{9} VEGF-D viral particles, had less than 50% stenosis. In contrast 4/8 of the animals that received the highest dose of virus had less than 50% stenosis at day 7. It is generally accepted that stenosis of less than 50% do not cause hemodynamic changes and are therefore regarded to insignificant in relation to flow restriction.

Table 7.5 shows the extent of stenosis measured at day 28. At day 28 all (8/8) of the results from the control animals and those that received 109 viral particles, had more than 75% stenosis. In contrast, only two out of the four animals that received the top dose of virus had more than 50% stenosis.

In addition to the ultrasound assessment, a microscopic evaluation of the site of anastomosis was performed in which the following parameters were assessed; thrombus, smooth muscle cell proliferation and endothelial proliferation; intimal proliferation, mural haemorrhage and thickening, medial smooth muscle cell proliferation, adventitial granulation tissue/fibroblast proliferation, perivenous haemorrhage/fibrinoid material, inflammatory cell infiltration, and granulation tissue/fibroblast proliferation.

At day three, at the site of anastomosis, there appeared to be a marginal increase in the degree of intimal proliferation in the animals that received the VEGF-D in comparison to the two control groups. Similarly, an increased incidence of medial smooth muscle cell proliferation and adventitial and perivenous granulation tissue/fibroblast proliferation were seen in the two groups which received the VEGF-D adenovirus the when compared to the control groups. These observations suggest that VEGF-D was having a biological effect as the site of anastomosis.

At day 14, endothelial proliferation and medial smooth muscle cell and intimal proliferation were seen only in the two groups that received the highest doses of the adenovirus containing the VEGF-D gene. Intimal proliferation/fibrosis was also seen in a single animal in the group which received the highest dose of virus, this animal also had a reduction in luminal diameter. However, in the animals treated with the VEGF-D transgene, the reduction in the luminal diameter was less in these animals than in the control animals. This observation suggests that VEGF-D may be having a beneficial biological effect. In addition, prominent adventitial neo-vascularisation with marked endothelial proliferation was only seen in the animals treated with the VEGF-D group, this also suggests that the VEGF-D protein was having a biological effect. A marginal increase in the degree of peri-venous granulation tissue/fibroblast proliferation was seen in the group treated with 10^9 viral particles when compared to the controls. There were no other notable differences between the four groups.

From the observations made at day 60, it was noted that there was an increased degree of intimal proliferation/fibrosis and a reduction in luminal diameter in the groups which received the VEGF-D adenovirus, when compared to the two control groups, and that luminal occlusion was seen only in animals treated with VEGF-D. Prominent perivenous blood vessels with medial smooth muscle cell proliferation were only seen in these animals, again indicating that VEGF-D expression had a biological effect. It is considered that an increase in the smooth muscle cell content may allow remodeling of the cell walls and that neo-vascularisation should increase oxygenation.

Clinical Monitoring: Monitoring of the animals during the 'in-life' phase of the study showed that no clinical signs attributable to the treatment regime were observed. Analysis of the bodyweight of the animals in the different groups showed that the increase observed in each of the different groups was comparable, and that the pattern of growth was typical for the age of the pigs used in the study. In addition, there was no difference in the consumption of food between the different groups. Similarly, there was no difference in the weights of the organs harvested from the treated and control animals at the time of autopsy. The majority of the adverse clinical signs that were recorded were considered to be a result of the surgical procedures that were performed.

Haematological and Biochemical Analysis: The results of the analysis of the haematological measurements, which assessed a variety of parameters including the white and red blood cell counts, showed that there was no difference between the animals treated with the adenoviruses and those treated with the saline controls. Similarly, no differences in the biochemical parameters, such as the level of alanine aminotransferase, aspartate aminotransferase, CPK iso-enzymes and lactate dehydrogenase were observed between the different groups.

Microscopic Analysis: Macro and microscopic analysis was performed on all the major organs harvested from each animal at the time of sacrifice. No differences were observed at the macroscopic between the treatment groups and the controls. In addition, histopathological analysis the different tissues showed that although there was evidence of infiltration of inflammatory cells in different organs, these cells were present in all the treatment groups and thus are not attributable to the gene therapy procedure. The analysis also showed that there was no significant toxicity in the liver or kidney in any of the treatment groups.

Immunocytochemical analysis: Immunocytochemical analysis, in which the number of macrophages at the site at which the collar was placed, showed that there was no difference in the number of macrophages observed in any of the treatment groups. However, the number of macrophages at the site of the anastomosis was higher in the samples collected from all groups at day 14 in comparison to the samples collected at day 3. As this observation was made in all groups, including the animals treated with the saline solution and suggests that the infiltration of the macrophages was not due to the adenovirus, but probably due to the breakdown of the collagen collar.

Degradation of the collar: Degradation of the collagen collar was recorded at the time that the animals were sacrificed. The results, given in Tables 7.2 and 7.3 show that at day 14, approximately 75% collar degradation was observed in most pigs and that at day 60, complete degradation of the collar was seen in all the animals.

Table 7.2: Collar degradation results from the animals terminated at day 14

Group	Pig no.	Degree of degradation (%)	Comments
Group 6 VEGF Dose 2	17m 1019m 18f 20f	75 75 75 75	Very slight amount of fluid around graft
Group 7 VEGF Dose 3	21m 23m 22f 24f	75 30 75 75	Shape maintained Copious fluid around graft and surrounding tissues
Group 8 Saline control	25m 26f	10 30	Very neat, very little fluid around the site
Group 9 Marker LacZ	27m 28f	50 50	

Table 7.3: Collar degradation results from the animals terminated at day 60

Group	Pig no.	Degree of degradation (%)	Comments
Group 10 VEGF Dose 2	129m 31m 30f 132f	100 100 100 100	Very hard material around venous anastomosis
Group 11 VEGF Dose 3	33m 35m 34f 36f	100 100 100 100	
Group 12 Saline control	37m 38f	100 100	
Group 9 Marker LacZ	39m 40f	100 100	

□ ***Discussion***

In conclusion, the VEGF-D PCR results from the samples collected from the target site of anastomosis, showed that the majority of the samples from the samples animals that received the two highest doses of virus were positive showing that adequate levels of transduction had been achieved. The RT-PCR results for the samples collected at the site of anastomosis from the animals which received the two highest doses of virus showed as expected, that expression of the transgene was transient and was predominately detected in the day 3 samples. Although the virus was widely distributed in the tissues harvested from outside the site of administration, RT-PCR analysis of these tissues showed that the transgene was only expressed in seven of the 494 tissues analyzed, suggesting that the level of transduction of these tissues was lower than at the site of anastomosis.

Analysis of the flow of blood by ultrasound, through the site of anastomosis 28 days post treatment, showed that in the pigs that received the highest dose of virus, no significant stenosis was observed in 50% of the animals (n = 4). In contrast, in the two control groups which received either saline, or an adenovirus carrying a marker gene, and the group with 10^9 viral particles of the VEGF-D adenovirus, the grafts were either not patent, or there was more than 75% stenosis. These results suggest that expression the VEGF-D transgene may delay the development of intimal hyperplasia at this time point. This conclusion is supported by the results of the microscopic analysis, at day 14, in which the incidence of the reduction in luminal diameter was less in the two groups of animals treated with the VEGF-D adenovirus. However, it should be noted that at day 60, no animals in the control group (n = 4) had an occluded lumen, whilst 3/8 of the animals which received the VEGF-D adenovirus had a luminal occlusion.

Analysis of the general health of the animals and the haematological and biochemical parameters showed that there were no adverse events associated with the surgery.

□ In summary, this study shows that treatment of domestic pigs with a dose of 10^{11} viral particles of the VEGF-D adenovirus reduced the level of graft stenosis, as assessed by ultrasound at day 28, when compared to animals that received either lower doses of the virus, a marker gene or saline. No immune toxicological responses were associated with any of the doses of adenovirus used.